

Function of the R Domain in the Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel*

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For a cystic fibrosis transmembrane conductance regulator (CFTR) channel to enter its open state, serine residues in the R domain must be phosphorylated by cAMP-dependent protein kinase, and intracellular ATP must bind to the nucleotide-binding folds and subsequently be hydrolyzed. CFTR with its R domain partially removed, $\Delta R(708-835)$ -CFTR, forms a chloride channel that opens independently of protein kinase A phosphorylation, with open probability approximately one-third that of the wild type CFTR channel. Deletion of this portion of the R domain from CFTR alters the response of the channel to 5'-adenylylimidodiphosphate, pyrophosphate, and vanadate, compounds that prolong burst duration of the wild type CFTR channel but fail to do so in the ΔR -CFTR. In addition, the addition of exogenous unphosphorylated R domain protein, which blocks the wild type CFTR channel, has no effect on the ΔR -CFTR channel. However, when the exogenous R domain is phosphorylated, significant stimulation of the ΔR -CFTR channel results; P_o increases from 0.10 to 0.22. These data are consistent with a model for CFTR function in which the R domain in the unphosphorylated state interacts with the first nucleotide binding fold to inhibit either binding or hydrolysis of ATP or transduction of the effect to open the pore, but when the R domain is phosphorylated, it undergoes conformational change and interacts at a separate site in the first nucleotide binding fold to stimulate either binding or hydrolysis of ATP or transduction of the effect to open the pore.

The cystic fibrosis transmembrane conductance regulator (CFTR)¹ belongs to a family of ATP-binding cassette transporters (1). Between two motifs in CFTR, each containing a membrane-spanning domain and a nucleotide binding fold (NBF), resides an intracellular regulatory (R) domain of ~240 amino acids (residues 590–830 encoded by exon 13) (2). CFTR constitutes a linear conductance chloride channel, which is regulated

by cAMP-dependent protein kinase (PKA) phosphorylation and by binding and hydrolysis of intracellular ATP (3, 4). The R domain is a special feature of the CFTR molecule, unique among members of the ATP-binding cassette transporter family. Phosphorylation of serine residues in the R domain is a prerequisite for opening of the CFTR channel. Only the phosphorylated CFTR channel can open in response to ATP binding and hydrolysis (5–8).

Rich *et al.* (9) demonstrated that deletion of 128 amino acids from the R domain of CFTR, $\Delta R(708-835)$, leads to a chloride channel that opens without PKA phosphorylation. This portion of the R domain corresponds to sequences that are not conserved in related proteins, *i.e.* MDR1 and STE6 (9–11). The portion of the R domain that remains in ΔR -CFTR corresponds to amino acids that are present in other ATP-binding cassette transporters between the predicted first nucleotide binding fold and the predicted second membrane-spanning domain (9, 11). Our studies show that unphosphorylated R domain protein synthesized *in vitro* (amino acids 590–858) interacts specifically with CFTR to inhibit chloride conductance in a phosphorylation-dependent manner (12). These studies were interpreted to be consistent with the hypothesis that the putative “gating particle” of the CFTR channel resides within the R domain.

The first and second NBF of CFTR share sequence similarity in certain conserved regions such as Walker A and Walker B motifs, but the overall amino acid homology between the two NBFs of CFTR is only ~30%. Functional studies of CFTR containing site-directed mutations in NBF1 and NBF2 suggest that the two NBFs have different roles in the gating of the CFTR channel (13–15). Mutations predicted to interfere with nucleotide hydrolysis in the first NBF reduce the channel opening rate, but the corresponding mutations in the second NBF result in prolonged channel openings (15). The use of compounds that alter the ATP hydrolysis cycle of CFTR, such as 5'-adenylylimidodiphosphate (AMP-PNP), PP_i, and vanadate (VO₄), provided evidence that hydrolysis of ATP is not only required for channel opening but also is involved in channel closure from the bursting state (16–19). Based on these studies, it was proposed that ATP hydrolysis at NBF1 initiates a burst of activity and that hydrolysis at NBF2 terminates a burst of open events (15).

This dual regulatory mechanism of the CFTR channel (PKA phosphorylation of the R domain and ATP binding and hydrolysis by the NBFs) requires coordinated interactions among the three intracellular domains: NBF1, R, and NBF2. However, little is known about the functional consequences of such interaction. It has been suggested that the rate of ATP hydrolysis by the two NBFs is sensitive to the degree of R domain phosphorylation (16), but it is not clear how the phosphorylation-induced conformational change in the R domain is connected

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¹ The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; NBF, nucleotide binding fold; PKA, cAMP-dependent protein kinase; AMP-PNP, 5'-adenylylimidodiphosphate; VO₄, vanadate; mAb, monoclonal antibody; RDP, R domain protein; pS, picosiemens(s).

with the binding and hydrolysis of ATP by the NBFs, which in turn controls channel opening and closing transitions.

To explore the potential interaction between the R domain and the NBFs in the function of the CFTR channel, we compared the single channel properties of the wild type CFTR and its R domain deletion mutant, $\Delta R(708-835)$ -CFTR. The wild type and ΔR -CFTR were expressed in HEK 293 cells, from which microsomal membrane vesicles were isolated for single channel studies using the bilayer reconstitution technique. Consistent with the early work by Rich *et al.* (9), we found that ΔR -CFTR formed a chloride channel that opens independently of PKA phosphorylation. The ΔR -CFTR channel had open probability approximately one-third that of the wild type CFTR channel, due to fewer, shorter openings, which could not be prolonged by agents that increase burst duration in the wild type channel: AMP-PNP, vanadate, and PP_i . Although the pore properties of the ΔR -CFTR channel were comparable with wild type, the addition of exogenous unphosphorylated R domain did not reduce open probability (P_o), as it does for the wild type channel. This observation casts doubt upon the hypothesis that the exogenous R domain acts as a gating particle. However, phosphorylated R domain increased the opening rate, but not the burst duration, of the ΔR -CFTR channel and increased P_o by 2-fold (20). These data are consistent with a model for CFTR function that postulates that the R domain interacts with NBF1 in at least two ways. In the unphosphorylated state, it inhibits ATP binding or hydrolysis or inhibits translation of these events into channel openings. In the phosphorylated state, the R domain stimulates either binding or hydrolysis of ATP or stimulates the transduction of the effect into the channel opening at NBF1.

EXPERIMENTAL PROCEDURES

Subcloning of CFTR Gene—The wild type CFTR cDNA was subcloned into an Epstein-Barr virus-based episomal eukaryotic expression vector, pCEP4 (Invitrogen, San Diego, CA), between the *NheI* and *XhoI* restriction sites (Fig. 1A). The mutant gene, $\Delta R(708-835)$, which lacks 128 amino acids between 708 and 835, was shuttled from pBluescript into pCEP4 by substituting the corresponding fragment in wild-type pCEP4 with the mutant one between *BspI* and *PmlI* restriction sites. The mutant clone (pCEP4($\Delta R(708-835)$)) was confirmed by restriction enzyme digestions.

Expression of CFTR in HEK 293 Cells—A human embryonic kidney cell line (HEK 293) was used for transfection and expression of wild type and mutant CFTR proteins. The cell line contains a vector, pCMV-EBNA, that constitutively expresses the Epstein-Barr virus nuclear antigen-1 gene product and increases the transfection efficiency of Epstein-Barr virus-based vectors. pCEP4(CFTR) and pCEP4($\Delta R(708-835)$ -CFTR) were introduced into the HEK 293 cells by the liposome-based gene transfection method. The detailed procedure has been described elsewhere (12, 21).

Immunoprecipitation/Western Blot Assay of CFTR—HEK 293 cells transfected with pCEP4(CFTR) or pCEP4($\Delta R(708-835)$ -CFTR) were grown to confluence in 75-cm² flasks (usually 48–72 h after transfection). Cells were washed three times with ice-cold phosphate-buffered saline and lysed with 1 ml of ice cold radioimmune precipitation solution (150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) in the presence of protease inhibitors (10 μ g/ml aprotinin, 10 μ g/ml pepstatin A, 5 μ M diisopropyl fluorophosphate) and spun at 48,000 \times g for 1 h at 4°C. Monoclonal antibody against human CFTR, either mAb 13-1 (specific for the R domain) or mAb 24-1 (specific for the C terminus) (Genzyme, Cambridge, MA), was added to 350 μ l of cell lysis supernatant solution and incubated on ice for 90 min. Antibody complexes were then precipitated with 20 μ l of protein G-agarose beads by incubating at 4°C for 30 min on a rocker. The beads were washed with Tris-buffered saline three times, and the bound proteins were solubilized with 20 μ l of gel sample buffer (200 mM Tris-Cl, pH 6.7, 9% SDS, 6% β -mercaptoethanol, 15% glycerol, 0.01% bromophenol blue) and loaded onto a 6% SDS-PAGE gel. The proteins were then transferred to a polyvinylidene difluoride membrane, probed with monoclonal antibodies (mAb 13-1 or mAb 24-1, diluted 1:250), developed with peroxidase-conjugated affinity-purified goat anti-mouse

IgG, and detected by chemiluminescence according to the manufacturer's recommendation (ECL kit, Amersham Corp.).

Preparation of R Domain Protein (RDP)—The R domain was cloned from the cDNA for CFTR by polymerase chain reaction and subcloned into pBluescript SKII as described previously (12). Sequence data matched that of exon 13 and 85 base pairs of exon 14 of wild type CFTR. Protein was expressed in rabbit reticulocyte lysate. In the presence of [³⁵S]methionine, the radioactive band on SDS-PAGE autoradiography was of appropriate molecular mass (~30 kDa). Translation of the protein with nonradioactive amino acids, followed by SDS-polyacrylamide gel electrophoresis and Western blot with anti-R domain antibody (mAb 13.1) showed a single band at this molecular mass. The nonradioactive protein, subjected to phosphorylation with exogenous PKA and radioactive ATP, showed a radioactive band that migrates slightly more slowly than unphosphorylated control in SDS-polyacrylamide gel electrophoresis. Based on these data, we identified the protein product as exogenous R domain and demonstrated our ability to phosphorylate this protein *in vitro* under standard conditions, as reported previously (12).

Every batch of R domain protein was confirmed by Western blot and by [³⁵S]methionine label of a parallel reaction mixture to produce R domain protein. By [³⁵S]methionine labeling and quantitation of radioactivity in the protein product, we determined the concentration of R domain protein in each batch, which ranged from 20 to 60 ng/ml. To test the effect of R domain protein on the CFTR channel, 20 μ l of either phosphorylated or unphosphorylated R domain protein was added to the *cis* (intracellular) chamber. Control was the rabbit reticulocyte lysate mixture with no translated protein or the lysate used to translate the proteins of the brome mosaic virus, one of which is of the same molecular weight as the R domain protein (12).

Isolation of Microsomal Membrane Vesicles from HEK 293 Cells—The HEK 293 cells transfected with pCEP4(CFTR) or pCEP4($\Delta R(708-835)$ -CFTR) were harvested and homogenized using a combination of hypotonic lysis and Dounce homogenization in the presence of protease inhibitors (5 μ M diisopropyl fluorophosphate, 10 μ g/ml pepstatin A, 10 μ g/ml aprotinin, and 10 mg/ml benzamide) (12, 21). Microsomal membrane vesicles were isolated after sequential centrifugation at 1,000 \times g and 100,000 \times g; resuspended in a buffer containing 250 mM sucrose, 10 mM HEPES-Tris (pH 7.2)) at a protein concentration of 3–7 mg/ml; and stored at -75°C until use.

Reconstitution of CFTR Channel in Lipid Bilayer Membrane—Lipid bilayer membranes were formed across an aperture ~200 μ m in diameter with a lipid mixture of phosphatidylethanolamine/phosphatidylserine/cholesterol (6:6:1). The lipids were dissolved in decane at a concentration of 40 mg/ml (12, 21, 22). The recording solutions contained the following: *cis* (intracellular, 1-ml volume), 200 mM KCl, 2 mM ATP, 1 mM MgCl₂, and 10 mM HEPES-Tris (pH 7.4); *trans* (extracellular, 3-ml volume), 50 mM KCl, 10 mM HEPES-Tris (pH 7.4). Unless otherwise indicated, recordings of a single CFTR channel with $\Delta R(708-835)$ -CFTR were always performed without PKA present in the recording solution, and those with wild type CFTR were always performed with a 50 unit/ml concentration of PKA catalytic subunit (Promega) present in the *cis*-solution. Because the agonists for CFTR channel (ATP or PKA) were only present in the *cis*-solution, this condition selected only CFTR channels that were oriented in the bilayer membrane in the *cis*-intracellular *trans*-extracellular manner.

The data presented in this study were obtained with four different preparations of microsomal membrane vesicles isolated from the HEK 293 cells transfected with $\Delta R(708-835)$ -CFTR cDNA. The total number of experiments was 130. For the wild type CFTR, the total number of experiments was larger than 400.

Analysis of Single Channel Data—Single channel currents were recorded with an Axopatch 200A patch clamp unit (Axon Instruments). Data acquisition and pulse generation were performed with a 486 computer and 1200 Digidata A/D-D/A converter (Axon Instruments). The currents were filtered at a cut-off frequency of 100 Hz through an eight-pole Bessel filter and sampled at 2.5 ms/point. Single channel data analyses were performed with pClamp software (Axon Instruments).

RESULTS

Partial Deletion of R Domain from CFTR Leads to a Chloride Channel That Opens Independently of PKA—The HEK 293 cells transfected with wild type CFTR cDNA or $\Delta R(708-835)$ -CFTR cDNA under the control of cytomegalovirus promoter expressed a large amount of CFTR proteins, detected in a Western blot (Fig. 1A). When microsomal membrane vesicles

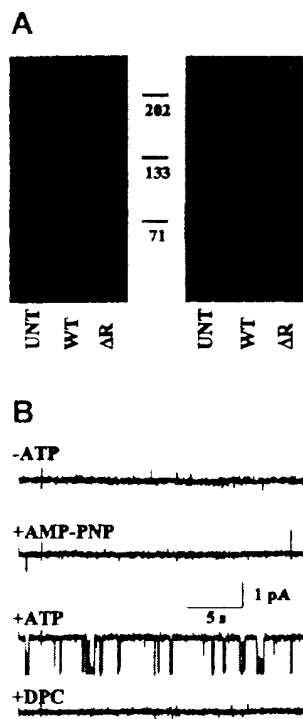


FIG. 1. Expression of wild type and $\Delta R(708-835)$ -CFTR in HEK 293 cells. pCEP4 eukaryotic expression vector containing the CFTR deletion mutant, $\Delta R(708-835)$, was generated by replacing the corresponding restriction fragment (*Bgl*I \rightarrow *Pml*I) in pCEP4(WT-CFTR) with that from the pBluescript($\Delta R(708-835)$ -CFTR). **A**, Western blots of HEK 293 cells transfected with pCEP4(CFTR) or pCEP4($\Delta R(708-835)$). Two monoclonal antibodies, mAb13-1, which recognizes the R domain, and mAb24-1, which recognizes the C terminus of CFTR, were used in the immunoprecipitation/Western blot procedure. mAb13-1 and mAb24-1 recognized mature fully glycosylated CFTR protein of 170 kDa in cells transfected with pCEP4(CFTR). $\Delta R(708-835)$ -CFTR lacks the epitope for mAb13-1; thus, it could only be detected with mAb24-1. **B**, selected single channel currents from a $\Delta R(708-835)$ -CFTR channel incorporated into the lipid bilayer membrane. Traces were acquired at a holding potential of -80 mV and obtained from a continuous experiment. The recording solution contained 200 mM KCl (*cis*-intracellular) and 50 mM KCl (*trans*-extracellular). The *cis*-solution contained 2 mM ATP with no PKA present. The concentration of diphenyl carboxylate was 3 mM.

isolated from these cells transiently expressing the wild type and mutant CFTR proteins were fused with the lipid bilayer membrane, functional chloride channels were routinely detected (Fig. 1B). The wild type CFTR formed a linear conductance chloride channel with slope conductance of 8.2 ± 0.6 pS in 200 mM KCl, the opening of which requires absolutely the presence of ATP (2 mM) and PKA (50 units/ml) in the *cis*-intracellular solution (12, 21, 23). The wild type CFTR channel exhibits slow kinetics of gating with mean open lifetimes of $\tau_{o1} = 11.8$ ms and $\tau_{o2} = 189.6$ ms (Fig. 2A) and an average P_o of 0.364 ± 0.042 at -80 mV (Fig. 2B).

The $\Delta R(708-835)$ -CFTR also formed functional chloride channels in the lipid bilayer membrane (Fig. 1B). These channels were linear with slope conductance of 8.0 ± 0.6 pS. Opening of the ΔR -CFTR channel still requires the hydrolysis of ATP, since AMP-PNP (0.5–4 mM) alone is insufficient to induce opening. Similar to the wild type CFTR, the ΔR -CFTR channel was sensitive to blockade by diphenyl carboxylate added to the extracellular solution (Fig. 1B). The ΔR -CFTR channel remained open, even in the absence of PKA phosphorylation, but with average P_o in 2 mM intracellular ATP of 0.122 ± 0.012 , about one-third that of the wild type channel (Fig. 2A).

To test whether PKA phosphorylation can enhance the activity of the ΔR -CFTR channel, experiments were performed with the same channel before and after the addition of PKA to the intracellular solution. As shown in Fig. 3, there were no changes in P_o following the addition of PKA (50 unit/ml concentration of the catalytic subunit) in seven paired experiments. To test whether the ΔR -CFTR channel had no response to PKA because it was phosphorylated *in vivo* by tonic PKA activity, we treated the channel with protein phosphatase 2A, (Promega), which reduced P_o of the fully phosphorylated wild type channel from over 0.31 to 0.02 within 6 min in seven paired experiments. There was no change in P_o of ΔR -CFTR following protein phosphatase 2A treatment for over 20 min ($n = 4$). Thus, neither PKA phosphorylation nor PP2A dephosphorylation changed the P_o of ΔR -CFTR. This channel was not regulated by phosphorylation.

Further single channel data analyses revealed different gating properties between the wild type and ΔR -CFTR channels (Fig. 2). First, the transition rate into the open state was reduced in ΔR -CFTR, since the number of open events was significantly less (compare the plateau phase of the cumulative open time histograms in Fig. 2B). Second, the distribution of open lifetimes was different (Fig. 2B). The cumulative open time histogram of the wild type CFTR channel could be fitted with two exponential functions with time constants of 12 and 190 ms, whereas the ΔR -CFTR channel could only be fitted with one exponential function with a time constant of 78 ms. Thus, it appears that the wild type CFTR channel contained two open states and that the ΔR -CFTR channel contained only one open state, which can be resolved at a time resolution of 60 Hz cut-off frequency.

Thus, the ΔR -CFTR channel is not regulated by PKA phosphorylation, although a phosphorylation site known to be used in wild type CFTR, S660, remains intact in ΔR -CFTR. Since the P_o of ΔR -CFTR channel is low even in the presence of PKA, we postulated that the decreased opening rate of the ΔR -CFTR channel (Fig. 2B) is due at least in part to alterations in the ATP-dependent gating of the CFTR channel.

AMP-PNP Does Not Stabilize the Open State of the ΔR -CFTR Channel—AMP-PNP, a nonhydrolyzable analogue of ATP, stabilizes the open state of the phosphorylated wild type CFTR channel in the presence of ATP (16, 17, 19, 24). In the presence of 2 mM ATP, AMP-PNP (0.4–5 mM) induced long open states in the wild type CFTR channel expressed in HEK 293 cells (Fig. 4A), with an increase in mean open lifetime from 96.0 ± 9.3 ms to 133.2 ± 15.6 ms (Fig. 4B). In contrast, AMP-PNP (2 mM) had no effect on either the open lifetime or the P_o of the ΔR -CFTR channel (Fig. 4B). The effect of AMP-PNP on ΔR -CFTR was independent of PKA phosphorylation (data not shown). Higher concentrations of AMP-PNP (>10 mM) inhibited the opening rate of both wild type and ΔR -CFTR channels (data not shown).

PP_i Fails to Prolong the Open State of the ΔR -CFTR Channel—We next tested the effect of PP_i on the wild type and ΔR -CFTR channel. PP_i had a biphasic effect on the wild type CFTR channel (Fig. 5). At 0.2–5 mM, PP_i increased P_o of wild type CFTR by promoting long open states of the channel (Fig. 5, left panels), but at concentrations >10 mM, PP_i reduced P_o . In contrast, the stimulating effect of PP_i was not seen in the ΔR -CFTR channel (Fig. 5, right panels). In more than 14 experiments, we never observed any increase in P_o or burst duration of the ΔR -CFTR channel following the addition of PP_i (0.2–10 mM). Rather, PP_i, even at low concentrations, consistently inhibited the opening rate of the ΔR -CFTR channel (Fig. 5B). At very high concentrations of PP_i, both the CFTR and the ΔR -CFTR channels opened predominantly to the 3-pS conductance state, which complicated calculation of P_o .

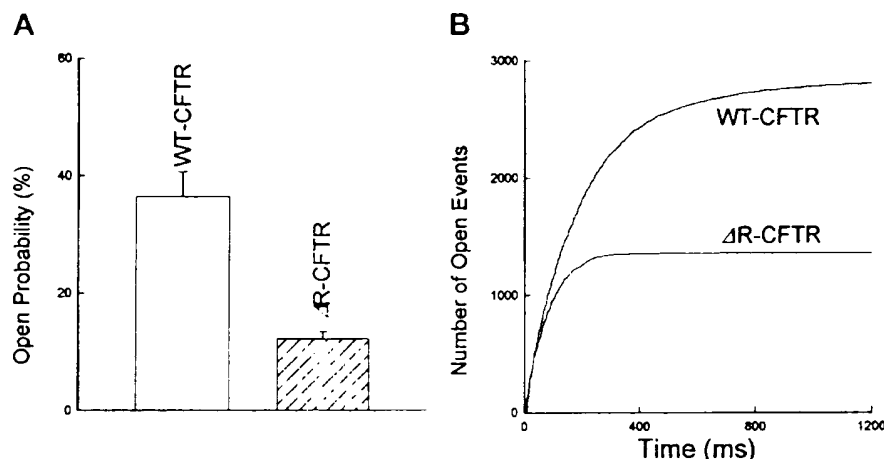


FIG. 2. Gating kinetics of the wild type CFTR and $\Delta R(708-835)$ -CFTR channels. **A**, single channel open probabilities (P_o) were calculated at -80 mV from a total of 36 single channel experiments with the wild type CFTR and 24 single channel experiments with the $\Delta R(708-835)$ -CFTR. The average values were $P_o = 0.122 \pm 0.012$ for the $\Delta R(708-835)$ -CFTR channel, and 0.364 ± 0.042 for the wild type CFTR channel. **B**, cumulative open time histograms at -80 mV. Open events were analyzed after 60-Hz digital filtering of single channel currents recorded at 100 Hz cut-off filtering frequency. 30 data files, each containing 25 s of continuous recording at -80 mV, were used for both the wild type CFTR ($n = 9$ bilayer experiments) and $\Delta R(708-835)$ -CFTR ($n = 8$) channels. A total of 2790 and 1362 open events were detected, respectively, for the wild type and $\Delta R(708-835)$ -CFTR channels. The histograms were fitted with the following equation: $y = W_{o1}(1 - \exp(-t/\tau_{o1})) + W_{o2}(1 - \exp(-t/\tau_{o2}))$. The best fit parameters were $W_{o1} = 232.3$, $\tau_{o1} = 11.8$ ms, $W_{o2} = 2557.8$, $\tau_{o2} = 198.9$ ms for wild type CFTR and $W_{o1} = 650.2$, $\tau_{o1} = 75.8$ ms, $W_{o2} = 712.0$, $\tau_{o2} = 78.2$ ms for $\Delta R(708-835)$ -CFTR.

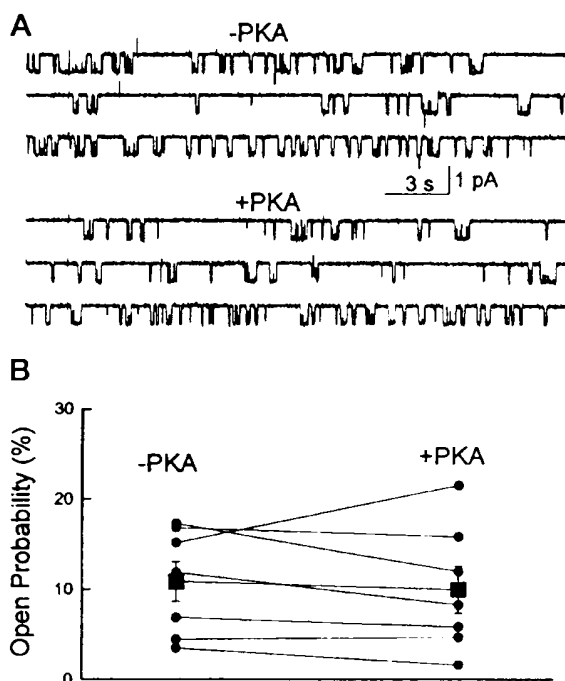


FIG. 3. ΔR -CFTR forms a PKA-phosphorylation independent chloride channel. **A**, selected single channel currents at -80 mV were taken from the same $\Delta R(708-835)$ -CFTR channel, before ($-PKA$) and after ($+PKA$) the addition of a 100 unit/ml concentration of the catalytic subunit of PKA to the *cis*-solution, which contained 2 mM ATP. **B**, channel open probabilities from seven paired experiments, in the absence and presence of PKA, were plotted. The averaged value did not change significantly following the addition of PKA; $P_o = 0.101 \pm 0.042$ without PKA and 0.099 ± 0.036 plus PKA.

It has been observed in several laboratories that the ability of CFTR to be locked open by PP_i and ATP analogs depends on the opening rate of the channel (16, 25). We also observed that the effect of PP_i and AMP-PNP on the wild type CFTR correlated with P_o of the channel; the lock-open effect is faster with a higher P_o channel (data not shown). If the long open states

were just a statistical probability that occurred with enough openings, then observing the ΔR -CFTR channel, with P_o about one-third that of the wild type channel, for a longer time period should eventually allow us to observe prolonged open states. We continued recording of the ΔR -CFTR channel in typical experiments either with or without PKA for 10 min without ever observing prolonged channel openings. After the addition of AMP-PNP (2 mM, $n = 6$) or PP_i (0.2–5 mM, $n = 17$), we recorded the channel for over 20 min and never observed the prolonged open state. The open time histogram demonstrating this for PP_i is displayed in Fig. 10. We conclude that the failure of the ΔR -CFTR channel to enter the bursting state, with or without these drugs, is not merely the consequence of low P_o and fewer openings of the channel but represents a distinct functional difference between wild type CFTR and ΔR -CFTR.

VO₄ Inhibits P_o of the ΔR -CFTR Channel—Vanadate, an analog of P_i, inhibits a variety of transport ATPases (26). It binds tightly at a site from which P_i is released following its cleavage from ATP and thus precludes further cycles of ATP hydrolysis. Vanadate enhances the activity of the native CFTR channel in the heart membrane (17) and the wild type CFTR channel heterologously expressed in different cell lines (19, 24). The effect of VO₄ on the wild type and ΔR -CFTR channels is shown in Fig. 6. Single channel records indicate that the stimulatory effect of VO₄ on the wild type CFTR channel is largely due to the increase in open lifetime. At VO₄ concentrations > 1 mM, the wild type CFTR channel remained open essentially all of the time ($n = 14$, Fig. 6, left). In contrast, VO₄ reduced P_o of the ΔR -CFTR channel ($n = 6$; Fig. 6, right).

Subconductance States of the Wild Type and ΔR -CFTR Channels—We have shown previously that the wild type CFTR channel contained two distinct subconductance states of ~ 5 –6 pS (M) and ~ 3 pS (L) in addition to the full conductance state (8 pS, H) (21–23). Under conditions of 200 mM KCl (*cis*)/50 mM KCl (*trans*) with no divalent cations present in the extracellular solution (0 mM Mg), both H and L states are measured in stable single channel recordings, and they slowly and spontaneously interconvert. The M state is rarely observed (23). Under these conditions, the subconductance states of the CFTR channel represent a reproducible property of the channel pore. We compared the subconductance states of the ΔR -CFTR chan-

FIG. 4. Effect of AMP-PNP on the wild type CFTR and ΔR -CFTR channel. Representative single channel currents were taken from two separate experiments with the wild type CFTR (WT-CFTR, left panel) and $\Delta R(708-835)$ -CFTR channel (middle panel), before (Control) and after the addition of AMP-PNP (+2 mM AMP-PNP) to the cis-solution. The arithmetic mean open lifetime (τ_{mean}) of the wild type (WT) CFTR channel increased from 96.0 ± 9.3 ms to 133.2 ± 15.6 ms following the addition of AMP-PNP ($n = 4$), while that of the $\Delta R(708-835)$ -CFTR channel did not change significantly. $\tau_{\text{mean}} = 68.7 \pm 5.2$ ms (Control), 66.1 ± 7.8 ms (+AMP-PNP).

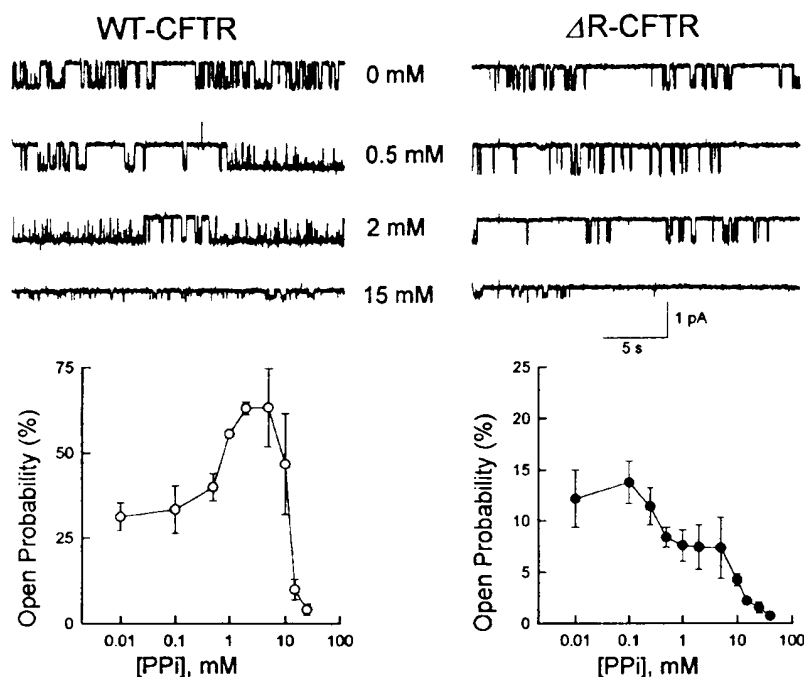
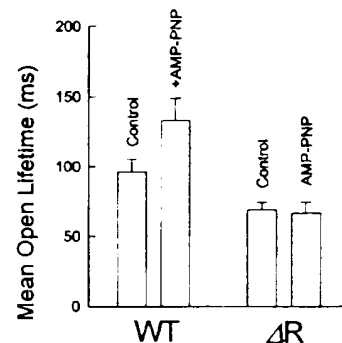
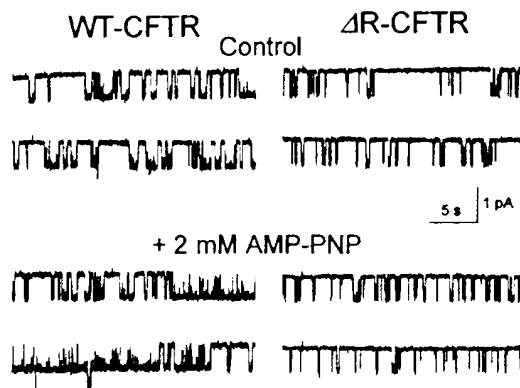
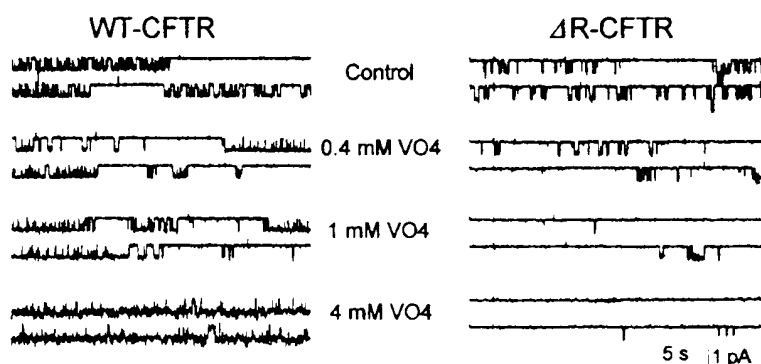


FIG. 5. Effects of pyrophosphate on the wild type CFTR (WT-CFTR) and ΔR -CFTR channel. Single channel currents were taken from two separate experiments with the wild type CFTR and $\Delta R(708-835)$ -CFTR channels, following the addition of different concentrations of PP_i to the cis-solution (upper panels). The average open probabilities were plotted as a function of $[PP_i]$ for the wild type CFTR (lower left panel) and $\Delta R(708-835)$ -CFTR (lower right panel). Each data point represents the average of 3–8 experiments. PP_i had a biphasic effect on the wild type CFTR channel, but it only had an inhibitory effect on the $\Delta R(708-835)$ -CFTR channel.

FIG. 6. Effects of vanadate on the wild type CFTR (WT-CFTR) and ΔR -CFTR channel. Representative single channel currents were obtained from a continuous experiment with the wild type CFTR channel (left panel) and the $\Delta R(708-835)$ -CFTR channel (right panel), following the addition of various concentrations of VO_4 to the cis-solution. VO_4 enhanced the activity of the wild type CFTR channel, but it inhibited the activity of the $\Delta R(708-835)$ -CFTR channel. Similar phenomena were observed in seven other experiments with the wild type CFTR channel and six other experiments with the $\Delta R(708-835)$ -CFTR channel.



nel with wild type to further test its pore properties. The 3-pS (L) subconductance state was also observed with the ΔR -CFTR channel. Fig. 7 shows consecutive single channel episodes obtained from a wild type (left panel) and a ΔR -CFTR channel (right panel), illustrating conversion of a single CFTR channel from the H to L conductance state. The L state of the ΔR -CFTR channel was similar to that of the wild type CFTR channel in terms of kinetics and current amplitudes, since both channels underwent fast transitions between the closed and partially open states once they occupied the L state. In a total of 74

successful experiments with the ΔR -CFTR channel, we observed 9 channels underwent transition from 8 to 3 pS, 6 channels underwent reverse transition from 3 to 8 pS, and 7 channels remained stable in the 3-pS conductance state without conversion to the 8-pS state within the time course of the bilayer experiments. The subconductance states of the ΔR -CFTR channel occurred at comparable frequency and converted with kinetics comparable with those of the wild type CFTR channel (23).

When the PP_i concentration was increased to above 15 mM

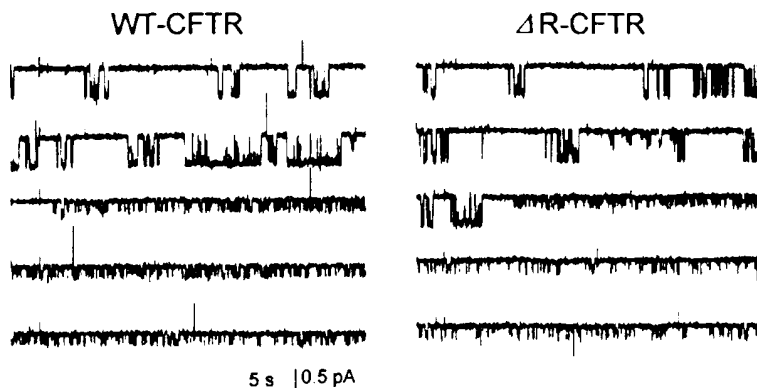


FIG. 7. Subconductance states of the wild type CFTR (WT-CFTR) and Δ R-CFTR channel. Both wild type CFTR and Δ R(708–835)-CFTR channels exhibit subconductance states of 3 pS, in addition to the full conductance state of 8 pS. The 8- and 3-pS conductance states interconvert within the same channel. Represented are 125 s of continuous recording at -80 mV for the wild type CFTR (left panel) and Δ R(708–835)-CFTR channel (right panel), demonstrating transition from the 8- to the 3-pS conductance states. Transitions from the 3- to the 8-pS states were also observed for both the wild type and Δ R(708–835)-CFTR channels (not shown). In a total of 74 experiments with the Δ R(708–835)-CFTR channel, 9 experiments showed transition from 8 to 3 pS, 6 experiments showed transition from 3 to 8 pS, and 7 channels remained stable in the 3-pS state without conversion to the 8-pS state during the course of the experiment. These conversion processes were not very different from those for the wild type CFTR channel (23).

(see the single channel traces at 15 mM PP_i , Fig. 5), both the wild type and Δ R-CFTR channels often (6 of 9 experiments, wild type; 6 of 10 experiments, Δ R-CFTR) underwent irreversible transition to a low conductance level (3 pS). These subconductance states represent openings of CFTR, since they can be completely blocked by 3 mM diphenyl carboxylate added to the extracellular solution but not by 300 μ M diisothiocyanostilbene disulfonate (data not shown), and they never occur in untransfected cells. Based on current-amplitude histogram analysis, the conductance value of the PP_i -induced 3-pS state was similar to the subconductance state of Δ R-CFTR and wild type CFTR observed under basal conditions (in the absence of PP_i).

Exogenous Unphosphorylated R Domain Fails to Block Δ R-CFTR Channel Activity, but Exogenous Phosphorylated R Domain Significantly Stimulates Channel Activity in Δ R-CFTR—The wild type CFTR channel is blocked completely by the application of unphosphorylated R domain protein to the intracellular side of the channel. Blockade is relieved by phosphorylation of the exogenous R domain protein, prephosphorylated R domain protein is incapable of channel blockade, and controls consisting of reticulocyte lysate with irrelevant translation product failed to affect the CFTR channel (12). However, exogenous R domain protein, from the same tube as can block the wild type channel, did not affect the activity of the Δ R-CFTR channel (Fig. 8). This was true both in the absence of PKA (while the Δ R-CFTR channel opens in response to ATP alone) and when the Δ R-CFTR channel has been phosphorylated by PKA followed by the addition of protein kinase inhibitor in concentrations sufficient to inhibit further PKA activity prior to the addition of the R domain protein. Thus, neither the phosphorylated nor the unphosphorylated Δ R-CFTR channel is blocked by exogenous unphosphorylated R domain.

However, when the R domain protein is phosphorylated, a marked increase in the P_o of Δ R-CFTR occurs (Fig. 8). This stimulation occurs when PKA is added to the intracellular chamber in the planar lipid bilayer assay when ATP and R domain protein are already present ($n = 2$) or when the R domain protein is prephosphorylated before the addition to the unphosphorylated Δ R-CFTR channel preparation in the planar lipid bilayer ($n = 4$). The open time histograms shown in Fig. 9 were obtained from the Δ R-CFTR channel in the absence (solid line) and presence (dotted line) of the prephosphorylated R domain protein, within the same recording period. The addition of the phosphorylated R domain protein did not change the

burst duration of the Δ R-CFTR channel but increased the number of open events, which accounts for most of the increase in P_o .

To further examine the failure of the Δ R-CFTR channel to enter the bursting state, we studied the effect of PP_i on the open time of the channel in the presence of the phosphorylated R domain. At a concentration of 2 mM, PP_i failed to increase the open probability of the Δ R-CFTR channel in the presence of phosphorylated R domain. Fig. 10 displays the open time histogram for these experiments. Each curve was best fit with a single exponential, for which the time constant was 58 ms for the Δ R-CFTR channel alone in this series of paired experiments (78 ms in other experiments), 89 ms for the Δ R-CFTR channel in the presence of phosphorylated R domain, and 84 ms in the presence of phosphorylated R domain and 2 mM PP_i . Thus, there was no significant change in time constant with the addition of phosphorylated R domain or pyrophosphate. In particular, there was no evidence for bursting behavior.

DISCUSSION

Our data show that the R domain deletion mutant of CFTR, Δ R(708–835), forms a functional chloride channel with normal conduction properties and subconductance states, which does not require PKA phosphorylation to open. The P_o of the Δ R-CFTR channel is about one-third that of the wild type CFTR channel, whether or not it is phosphorylated. Unlike the wild type channel, Δ R-CFTR cannot be blocked by exogenous unphosphorylated R domain or stimulated by AMP-PNP, PP_i , or VO_4 . However, the addition of phosphorylated R domain protein results in an increase of the P_o of the Δ R-CFTR channel to about two-thirds that of the wild type channel. Nevertheless, the burst duration remains brief.

The R domain of CFTR is highly hydrophilic, with $\sim 28\%$ of the amino acids being charged residues. Site-directed mutagenesis and peptide-sequencing studies have identified five serines (660, 700, 737, 795, and 813) as the major physiological targets for phosphorylation by PKA, and the two best phosphorylation sites (Ser⁷³⁷ and Ser⁸¹³) are located within the region deleted in Δ R(708–835) (27, 28). The multiple phosphorylation sites in the R domain are redundant, since mutation of any one of them to alanine or the mutation of two or three in various combinations did not affect cAMP-dependent regulation of the CFTR channel, while combined mutation of four of the serines, 660, 737, 795, and 813 (4SA), reduced the open probability of the

FIG. 8. Effect of the R domain on the ΔR -CFTR channel. A, sample traces from a ΔR -CFTR channel under control conditions, following the addition of RDP and following the addition of PKA to the *cis*-chamber containing the RDP. Records were filtered at 100 Hz. B, open probability of ΔR -CFTR channel. The first two bars show data from channels tested in the presence and absence of PKA. The second three bars show channels to which unphosphorylated R domain protein was added into the *cis*-chamber, with no change in open probability. However, when R domain protein was present, the addition of PKA increased the open probability approximately 2-fold.

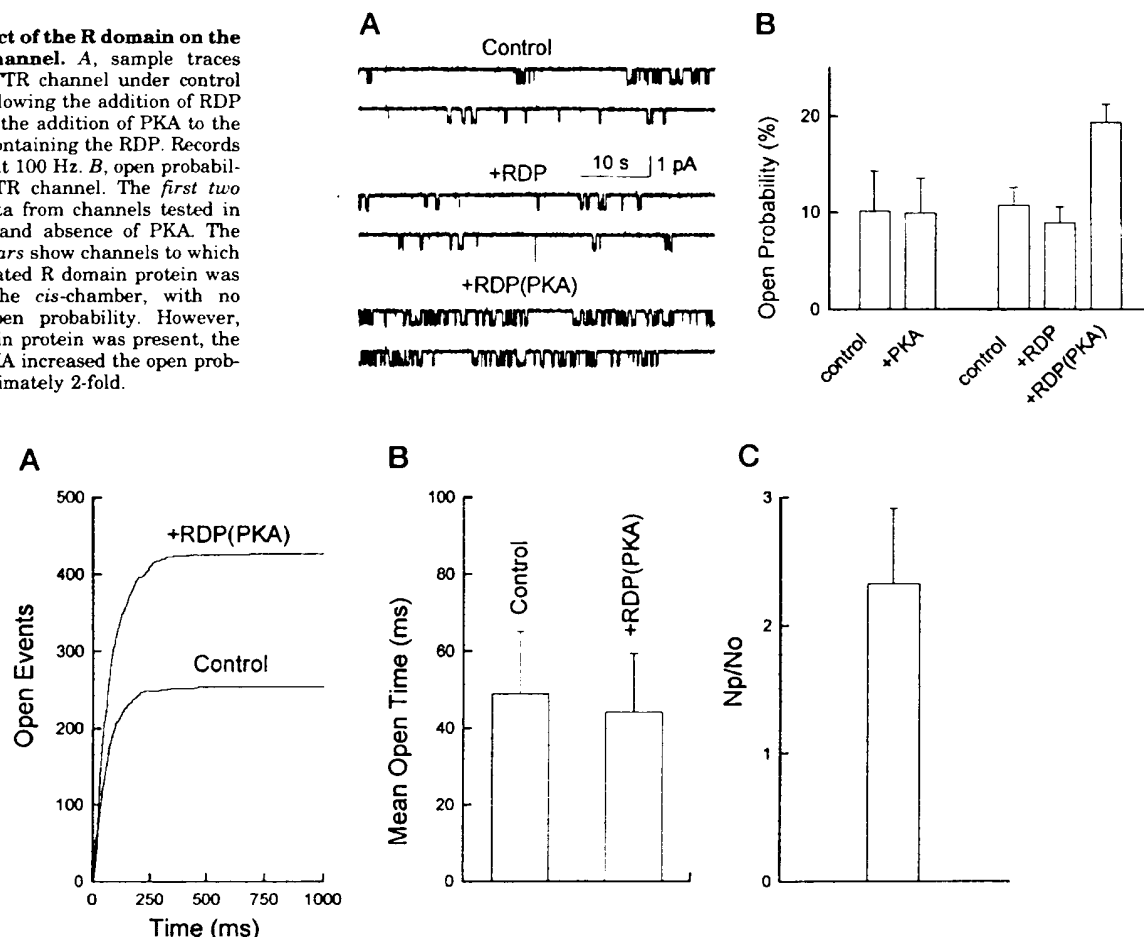


FIG. 9. Effect of phosphorylated R domain protein on the open lifetime of the ΔR -CFTR channel. A, cumulative open time histograms were generated from 150 s of continuous recording at -80 mV from a single $\Delta R(708-835)$ -CFTR channel, before (Control) and after the addition of prephosphorylated R domain protein (+RDP(PKA)) to the *cis*-solution. The histograms were fitted with the following equation: $y = N(1 - \exp(-t/\tau_o))$, where $N_o = 253.6$, $\tau_o = 60.3$ ms (control); and $N_p = 425.3$, $\tau_p = 70.6$ ms (+RDP(PKA)). B, the arithmetic means of open times (τ_m) were calculated from five paired experiments with the ΔR -CFTR channel before and after the addition of prephosphorylated R domain protein. $\tau_m = 48.9 \pm 16.2$ ms (control), 44.0 ± 15.4 ms (+RDP(PKA)). C, the number of open events was increased 2.32 ± 0.59 -fold (N_p/N_o), following the addition of prephosphorylated R domain protein to the *cis*-solution.

CFTR channel by nearly 50% (10). More extensive combined mutations of up to nine serine residues and one threonine residue (10SA) led to further decrease in channel activity, but the channel still remained tightly controlled by PKA and ATP (8). The remaining regulatory site is serine 753, not a dibasic consensus PKA phosphorylation site (29).

By studying the function of the single ΔR -CFTR channel in the bilayer membrane, we confirm some of the previous studies of Rich *et al.* (9); ΔR -CFTR forms a chloride channel that does not require PKA phosphorylation to open. However, Rich *et al.* (9) reported that chloride efflux from cells transfected with ΔR -CFTR increased in response to cAMP stimulation, whereas we found no effect of PKA phosphorylation on the ΔR -CFTR activity. This apparent inconsistency probably results from the different systems used for study. The planar lipid bilayer assesses the effect of phosphorylation on single channel activity, whereas in whole cells, increased chloride efflux could result from increased delivery of functional channels to the cell surface as well. The complete lack of response of the ΔR -CFTR channel to PKA phosphorylation, despite the retention of the Ser⁶⁶⁰ site, which is sufficient to stimulate channel openings if the other major phosphorylation sites are mutated, suggests that the large deletion from the R domain prevents it from assuming a stimulatory conformation when it is phosphoryl-

ated. It is unlikely that the failure of P_o to increase in response to PKA in the ΔR -CFTR channel is caused by active phosphatase activity, for in the same planar lipid bilayer system, when exogenous R domain protein is present, PKA causes P_o to double. Moreover, when protein kinase inhibitor is added to phosphorylated wild type channels contained in HEK 293 microsomal vesicles, no rundown is observed over 10–20 min of observation, suggesting that phosphatase activity in HEK 293 vesicle preparations is minimal (12). If phosphatase is added to ΔR -CFTR channels, there is no decrease in P_o , indicating that this channel is not unresponsive to PKA because it is already fully phosphorylated by tonic PKA activity in HEK 293 cells or vesicles.

The role of the R domain in CFTR channel function may be clarified by these results. One possibility is that the R domain functions as a gating particle for the CFTR channel. This hypothesis is consistent with the observation that unphosphorylated R domain, added to the phosphorylated CFTR channel, prevents chloride transport and that elimination of the R domain in the ΔR -CFTR construct results in an open channel *in vivo*. However, it seems unlikely that the R domain operates like the gating particle described in some of the voltage-dependent ion channels (30, 31). Even the phosphorylated CFTR channel requires ATP hydrolysis to open (3), and exogenous

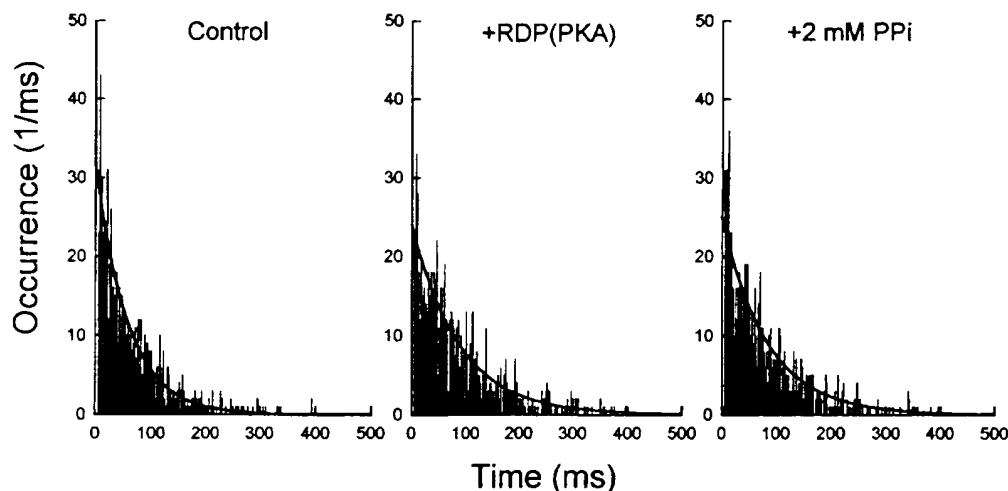


FIG. 10. Effect of PP_i on the ΔR -CFTR channel in the presence of phosphorylated R domain protein. Open time histograms were generated from 282 s of continuous recording at -80 mV from a single ΔR -CFTR channel, in the absence of PKA (Control), after the addition of prephosphorylated R domain protein (+RDP(PKA)) and after the addition of PP_i (+2 mM PP_i). Data were pooled from three paired experiments. Mean open probabilities for these channels were 0.142 (Control), 0.244 (+RDP(PKA)), and 0.204 (+2 mM PP_i). P_o values for the two samples in the presence of RDP(PKA) are significantly greater than P_o (Control). Solid lines represent the best fit according to the equation $y = W/\tau(\exp -t/\tau)$, where $W = 1843$ (Control), 2128 (+RDP(PKA)), or 2095 (+2 mM PP_i) and $\tau = 57.7$ ms (Control), 88.5 ms (+RDP(PKA)), and 83.5 ms (+2 mM PP_i).

unphosphorylated R domain fails to block ΔR -CFTR, although its pore properties appear normal. On the other hand, the unphosphorylated R domain must prevent gating by ATP, because channels with an intact R domain (wild type) (5), or even an R domain containing up to nine serine to alanine mutations (8), do not open in the presence of ATP until activated by PKA. The nonhydrolyzable ATP analog 8-azido-ATP binds equally well to phosphorylated and unphosphorylated CFTR channels (32), suggesting that the unphosphorylated R domain does not interfere with ATP binding to the NBFs, although these studies (32) do not address the stoichiometry or sites of ATP binding. However, when a substantial portion of the R domain is deleted, either an inhibitory portion of it is also deleted or the conformation of R changes so that it can no longer inhibit. Although the mutant channel opens in the absence of PKA phosphorylation, channel kinetics are abnormal; P_o is low and openings are fewer and briefer compared with wild type channels.

One possible explanation for briefer openings is that the channel closes more quickly in the ΔR -CFTR mutant. One current hypothesis for the regulation of channel closure is that binding and hydrolysis of ATP at the second NBF effect channel closure, particularly from the bursting state. Several pieces of evidence suggest that NBF2 has an inhibitory role in the function of the CFTR channel. Sheppard *et al.* (33) showed that the N-terminal half of CFTR (D893X, a molecule without NBF2) forms an ATP-regulated chloride channel that opens in the absence of PKA phosphorylation and, once phosphorylated, exhibits higher P_o than the full-length CFTR channel (see Fig. 3 of Ref. 33). Point mutations within the conserved Walker A motif of NBF1 decreased the opening rate of the CFTR channel, while the corresponding mutations in NBF2 (K1250A, K1250M) prolong the open lifetime of CFTR (14, 15). The functional effects of K1250A and K1250M on the CFTR channel are similar to the effects of AMP-PNP and PP_i (19, 24), suggesting that a decrease in the ATP hydrolysis rate at NBF2 leads to prolonged opening of the CFTR channel. To test whether inhibiting activity at the NBF2 in the ΔR -CFTR mutant increased the P_o , we applied several drugs that in wild type CFTR prolong burst duration, presumably by inhibiting ATP binding or hydrolysis at NBF2. However, neither AMP-PNP, PP_i , nor VO_4 increased P_o or burst duration in the ΔR -CFTR

mutant. Thus, it is unlikely that the low P_o in this mutant is the result of increased inhibitory activity of NBF2. Alternatively, it may be that the ΔR -CFTR channel never achieves either high enough P_o or the bursting state from which it can be closed by binding and hydrolysis of ATP at NBF2, so changing the functional capacity of NBF2 has no effect on this channel. However, increasing P_o by the addition of phosphorylated R domain to 0.24 did not confer on PP_i the ability to increase burst duration, so the lack of response to this agent in the ΔR -CFTR channel is probably not solely the result of low P_o .

An alternative hypothesis is that in the ΔR -CFTR mutant, a normal stimulatory effect of the phosphorylated R domain has been eliminated. To test this possibility, we added phosphorylated exogenous R domain protein to the ΔR -CFTR channel in the planar lipid bilayer. This maneuver resulted in a nearly 2-fold increase in P_o . Similar observations were made by Winter *et al.* (34). This increase resulted entirely from an increased number of channel openings, not increased burst duration. In addition, even when it was stimulated by exogenous phosphorylated R domain, the ΔR -CFTR channel did not respond to PP_i with an increase in P_o or burst duration (time constant).

Our data and the data of others are consistent with a model for R domain function in which the unphosphorylated R domain exerts strong inhibition on either the ATP binding or hydrolysis or the transduction of their effect to open the channel pore. However, when the R domain is phosphorylated, it either stimulates ATP binding or hydrolysis or enhances transduction of their effect to open the channel. The inhibitory effect of R domain protein is relieved by phosphorylation, but if unphosphorylated R domain is available in excess, free in solution, inhibition can be effected. When a large portion of the R domain is deleted from CFTR, either the inhibitory portion is eliminated or the conformation of the remaining R domain is altered so that the inhibitory conformation cannot be assumed. The binding site for the unphosphorylated R domain is inaccessible to exogenous R domain in this configuration, possibly because the conformational change that occurs with phosphorylation is required to expose the inhibitory site to the solution or possibly because of constraints on the remaining amino acids in the shortened R domain channel. However, the ΔR -CFTR molecule, while lacking inhibition from the unphosphorylated R domain, also lacks the stimulatory effect of phosphorylated R

domain. This effect can be reconstituted in part by exogenous phosphorylated R domain: this stimulatory site is accessible to occupation by exogenous protein in the Δ R-CFTR channel but not in the phosphorylated wild type channel, in which we speculate that endogenous phosphorylated R domain has the structural advantage. Exogenous phosphorylated R domain increases the opening rate but not open time. Bursting behavior may require a more exact conformational fit than exogenous R domain can supply. Even the addition of PP_i to the Δ R-CFTR channel in the presence of phosphorylated R domain does not increase open time.

The conformation of the R domain, as assessed by circular dichroism, changes with phosphorylation in solution *in vitro* (35). Such a change in conformation might allow the phosphorylated R domain to interact at a different site on NBF1 or in a different manner from the less charged, unphosphorylated version. The search for interaction among the protein domains of CFTR by physical means has been frustrating, but Price and co-workers (36) report interaction between NBF1 and the R domain. Our model predicts that interactions might also be detected between NBF1 and phosphorylated R domain.

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